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13. ABSTRACT (Maximum 200 Words) Drug resistance is a process that occurs in a variety of carcinomas and especially in epithelial breast carcinomas. These carcinomas represent ~80% of all breast cancer types and are the subject of intense study. The origins of drug resistance in these cells are poorly determined. This proposal deals with examining the roles of the cell membrane and the properties of ion channels within this membrane in drug resistance. It is well known that the plasma membrane, through its role as a permeability barrier that defines and differentiates the intracellular from the extracellular one, plays a vital role in cell viability and survival to various noxious agents. However, the transport properties of breast epithelial cells and certainly those of cancerous origins are essentially undetermined. We propose to define these properties and to test the effects of transport alterations on cell viability and resistance to anthracycline antibiotics, agents which are widely used to combat breast cancer. Moreover, the routes of drug entry and exist across polarized cells (with apical and basolateral membranes) will be determined.				
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Introduction:

Epithelial breast carcinoma is a devastating and costly disease. A primary course of therapy involves the use of anthracycline antibiotics. While these agents provide a methods for combating the progression of some tumors, this treatment is highly susceptible to failure brought about by a process termed "drug resistance". This process has been historically attributed to the presence of drug efflux molecules that actively transport chemotherapeutics against a concentration gradient. One such transporter is P-glycoprotein or P-gp1. This transporter belongs to a class of membrane bound integral proteins termed ABC transport proteins. A common feature among these proteins is their ATP binding capacity. A membrane of this ABC transport proteins, CFTR (Cystic Fibrosis Conductance Regulator) is also an ion channel. Indeed, CFTR is well known to code for a plasma membrane Cl^- channel. Thus, this raises the possibility the P-gp1 might also code for an ion channel, and that its channel activity might be responsible, in part, for drug resistance.

This raises the question of what type of ion channels are present in the membrane of breast epithelial cells and specifically breast epithelial carcinoma cells? This is important as it pertains to the idea that the plasma membrane and its resident channels and transporters affect cell homeostasis and secondarily could also affect drug resistance. This is substantiated by recent data indicating that MCF-7 cells, a model for breast carcinoma, contains message (mRNA) for an Epithelial Na^+ Channel or ENaC. Moreover, ENaC message was more than eight fold increased in drug resistant MCF-7 or MCF-7Adr. This has lead us to examine the presence of ENaC proteins and functional Na^+ channels in MCF-7 cells. As ENaC and CFTR are known to interact with each other and affect each other's activity, we developed a hypothesis that tests the possibility that ENaC's function is important to cell viability and also drug resistance. The mechanisms by which ENaC affects these properties are unknown but were proposed to occur via effects on the intracellular environment, or via potential interactions between ENaC and P-gp1 akin to what occurs between ENaC and CFTR.

Besides, potential roles for P-gp1 and ENaC, other mechanisms by which drugs enter and leave polarized epithelial cells are also poorly defined. These include membrane diffusion, fluid phase endocytosis/exocytosis and other undefined means by which anthracycline antibiotics can cross the membrane. The dependence of these mechanisms on ENaC and P-gp1 function are also unknown. These issues need to be carefully defined before one can begin to understand the routes of drug entry and exit, and before attempting to manipulate such mechanism to increase drug retention within the cells.

Results/Body:

As mentioned in the previous reporting period we have identified the conditions which allow us to culture MCF-7 cells on permeable support and allow for cell polarization. This has allowed us to study ion transport at the whole epithelium level and at the single cell/single

channel levels. Utilizing electrophysiological techniques we demonstrated the presence of functional epithelial Na^+ channel (ENaC) in the apical membrane of these cells. This channel was also documented at the protein and mRNA levels. Utilizing these findings we set out to test the effects of channel function on daunomycin accumulation and efflux. However, before such experiments were carried out, we determined the routes of drug entry and exit.

Task 1: To test the hypothesis that an MDR phenotype is accompanied by changes of Na^+ -transport and to determine whether this is mediated by changes of ENaC protein levels or channel activity.

a. Characterization of membrane ionic channels

This was accomplished in the previous reporting period.

b. Effects of channel blockade on drug accumulation and/or resistance

b1: Mechanisms of drug uptake and efflux.

b2: Effects of ENaC on drug uptake and efflux

To address the two sub-aims above we determined apical and basolateral membrane daunomycin uptake and efflux. These values were also compared with the uptake and efflux of horse radish peroxidase (HRP). These values are commonly used as a reflection of fluid phase endocytosis and exocytosis. By comparing the fluxes of ^3H -daunomycin with those of HRP, we were able to assess the non-specific movement of this antibiotic across either the apical or basolateral membranes. As shown in Fig. 1, HRP uptake was highly temperature dependent- a finding entirely consistent with our understanding of vesicular trafficking in mammalian cells. An interesting finding was that uptake did not reach saturation even at time points as long as 20 h (1200 min). This indicates the presence of multiple compartment in which endocytosed vesicles can be routed to. Moreover, uptake across the basolateral membrane was ~ 2-4 fold higher at all

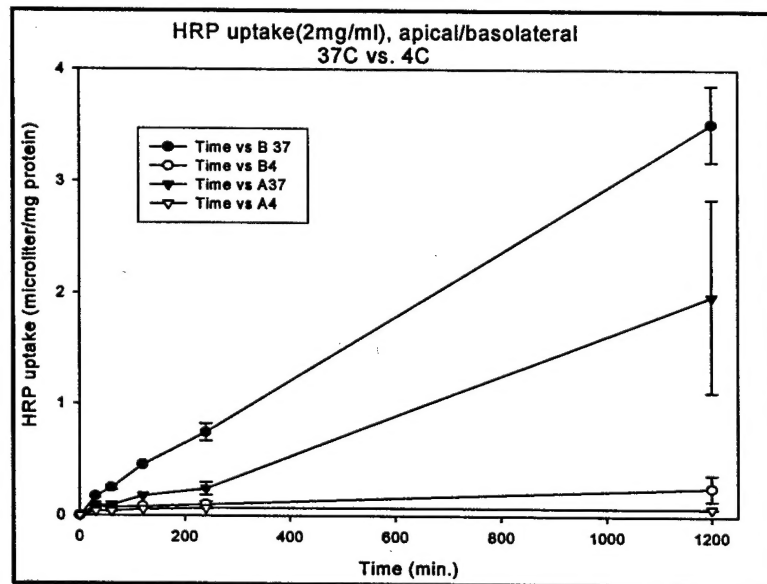


Figure 1. Fluid-Phase endocytosis across polarized MCF-7 cells. Uptake at 40C was essentially negligible, consistent with that expected in mammalian cells. This uptake is taken as an index of non-specific membrane binding and loading artifacts.

time points, consistent with the larger area (capacitance) of that membrane. This difference remained even at time points of longer than 24 h (data not shown), indicating distinct apical and basolateral vesicle compartments. When uptake data were converted back to volume per number of cells, we find that fluid phase endocytosis was on the order of $\sim 1 \mu\text{l}$ per 10^6 cells. This value is entirely consistent with the expected volume of 10^6 cells and when combined with the effects of 4°C , indicate that HRP can be successfully used as a marker of endocytosis in these polarized cells.

To compare uptake of ^3H -daunomycin with that of HRP, cells were exposed at 37°C to apical or basolateral solutions containing $15 \mu\text{M}$ daunomycin. As shown in Fig. 2, daunomycin uptake can be approximated by a first order saturatable function. In both apical and basolateral loading, uptake was found to saturate within ~ 60 min. As observed with HRP, basolateral loading was ~ 2 fold higher than apical. The data shown in fig.2 are converted to daunomycin volume, given the loading solution's concentration. Data are also calculated per tissue culture insert, which is equivalent to ~ 1 mg protein. Thus, we can directly compare the findings from figs. 1 and 2. It is clear that daunomycin accumulates in these cells at much higher rates (>100 fold) than can be accounted for by fluid-phase endocytosis. Given the differences in rates and saturation we can clearly rule out fluid-phase endocytosis as a contributor to apical or basolateral uptake. Moreover, given the much higher

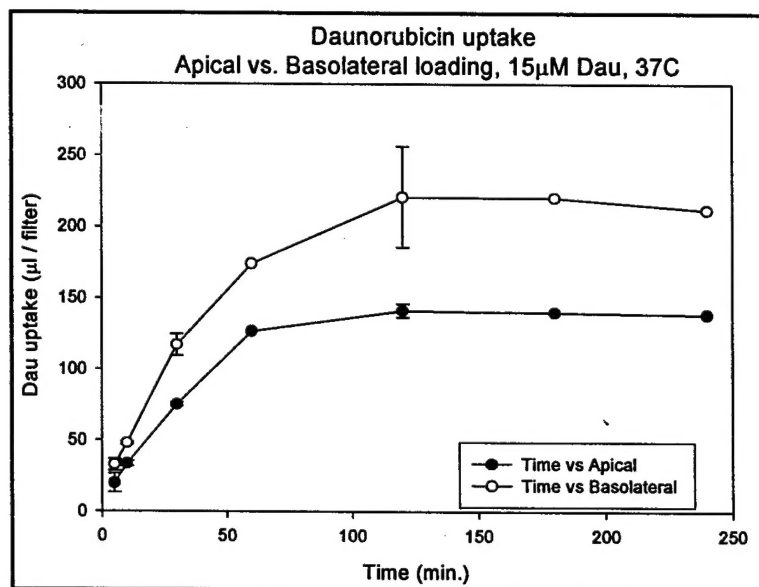


Figure 2. Daunomycin uptake across polarized MCF-7 cells. Data were normalized to the loading [^3H -daunomycin], and converted to μl uptake to allow comparison with HRP.

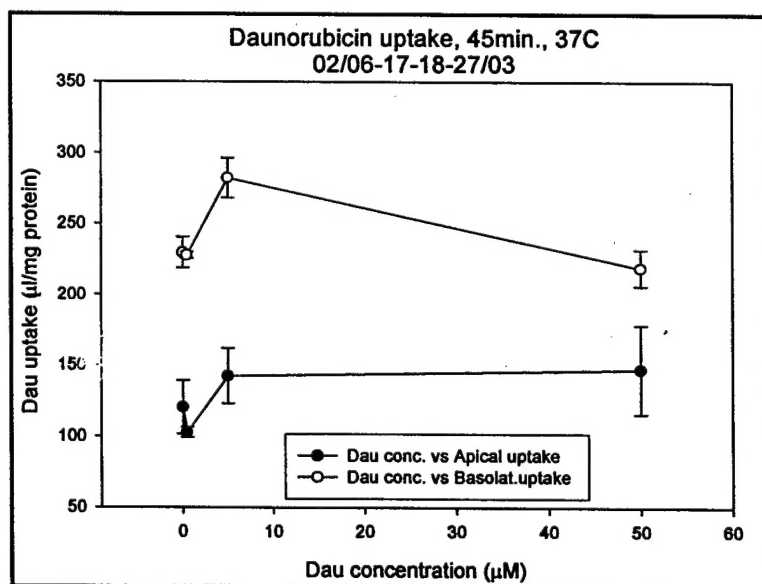


Figure 3. Uptake of Daunomycin is not concentration dependent.

volume of accumulation (100-200 μl), this also indicates a higher concentration inside the cells than that found in the loading solution, and rules out a simple diffusion process across the membrane as the sole mechanism of drug uptake. This is an important finding that here-to has not been previously described, and is interpreted as accumulation of Daunomycin against a concentration gradient..

To determine whether uptake is concentration dependent, we carried out experiments with apical and basolateral loading solutions with varying daunomycin concentrations (0.05 to 50 μM). As seen in Fig. 3, only small differences were observed over a four decade range of concentration. Interestingly, a paradoxical effect is observed between 0.5 and 5 μM whereby a higher accumulation level is observed, even after normalizing to the concentration of the loading solution. Taken together, these data indicate that uptake of anthracyclines is not a strictly passive process. This opens up new avenues of therapeutic research examining ways to modulate (enhance) uptake rather than, or in conjunction with, agents that inhibit efflux.

To further elucidate the nature of the uptake pathway, we examined the effects of temperature on this phenomenon. As shown in Fig. 4, a decrease of temperature from 37°C to 24°C results in a large inhibition of both apical and basolateral uptake. A further small inhibition is observed with a decrease to 4°C,

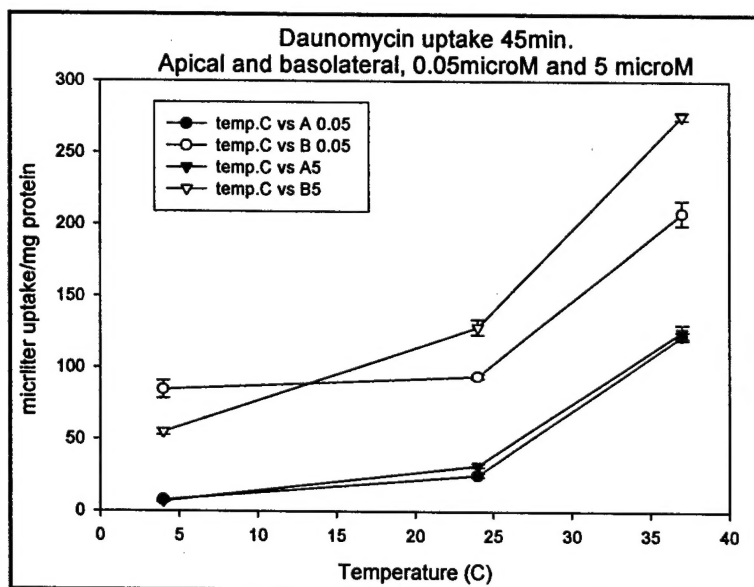


Figure 4. Effects of temperature on Daunomycin uptake.

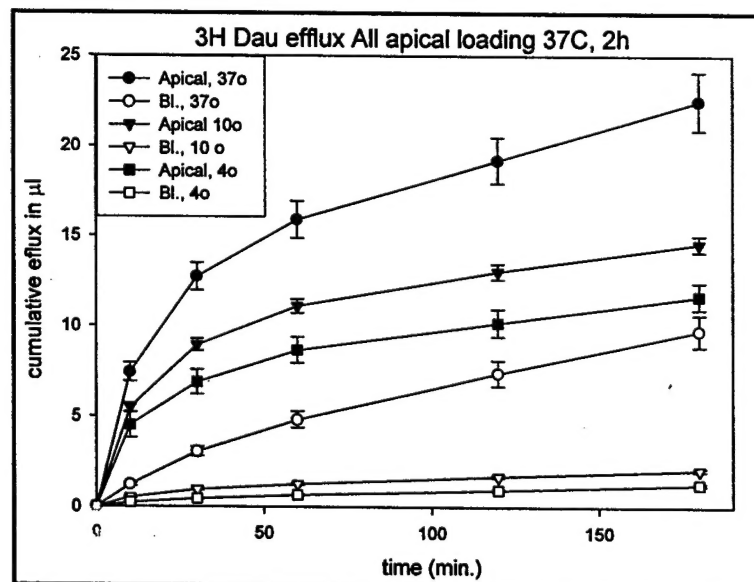


Figure 5. Effect of temperature on Daunomycin efflux

however, the majority of this effect is observed with the change to 24°C. These large changes are not consistent with simple diffusion, as most of diffusion related processes exhibit a Q_{10} in the range of 1.2-13. These finding provide further evidence for the hypothesis proposed above implicating a transporter in drug uptake.

To test whether similar effects are also observed with drug efflux, we examined the effects of temperature on daunomycin efflux in cells pre-loaded at 37°C. As shown in Fig. 5 smaller effects were observed with apical efflux in the temperature range of 37 to 4°C. This is likely a reflection of differences in the mechanisms responsible for cell loading and efflux. A similar conclusion can also be observed when examining the magnitude of drug accumulation and efflux, where it is clear that much larger levels can be accumulated within the cells in a time period of < 1h than that which can be extruded from these cells in more than 3 h. This again points to the importance of studying accumulation as means of improving drug efficacy.

Task 2: To test the hypothesis that an MDR phenotype can be induced with changes of Na^+ -transport and to determine whether this is accompanied by differences in P-gp1 protein levels or turnover rates.

We have attempted to create cells that are anthracycline resistant. This was carried out by gradually increasing the dose of anthracycline in the culture media. A second approach was to create cells that are TNF α resistant as those have been previously reported to exhibit altered resistance to anthracyclines. We have been able to create both cells subtypes. However, in both cases, the cells loose their ability to polarize and therefore we are not able to study the accumulation and efflux of these drugs separately in the apical and basolateral membranes. Moreover, in these non-polarized cells we are not able to functionally assess the levels of ENaC expression to attempt to correlate resistance with Na^+ channel activity. To circumvent this problem we have resorted to siRNA technology to decrease ENaC expression in polarized cells to study this effect on drug kinetics. Additionally, we plan to use GeneGun technology to transfer additional ENaC DNA to already polarized MCF-7 cells to documents effects on enhanced channel expression on Daunomycin uptake and efflux. These are important experiments that will allow us to alter expression in polarized cells, a procedure which is very difficult with conventional gene transfer methods. These experiments are currently underway and we expect to complete these by the end of the upcoming reporting period.

Task 3: To determine the mechanism of interaction between Na^+ -Transport and MDR

We have also carried out preliminary experiments in the *Xenopus* oocyte expression system to test the effects of P-gp1 expresison on ENaC. We find that similar to CFTR, co-expression of P-gp1 is able to downregulate ENaC activity. Experiments are currently underway with tagged ENaC subunits to determined whether this observation is due to decreased ENaC protein levels. We expect to finish these experiments in the upcoming year.

Key Research Accomplishment:

Determination of fluid phase endocytosis
Determination of cell exocytosis
Determination of polarized drug uptake and efflux.
Determination of the effects of temperature on uptake and efflux.
Further assessment of the effects of Na⁺ transport on drug efflux.
Development of a new hypothesis for transporter-mediated drug uptake.
Development of 2 cell lines and their use for studying drug uptake and efflux and Na⁺ transport.
Co-expression of P-gp1 and ENaC in oocytes and the demonstration that like CFTR, this results in inhibition of Na⁺ transport.

Reportable Outcomes:

1. Invited presentation, Tulane University Department of Physiology 2002.
2. One manuscript describing the electrophysiological data is in revision, and a second manuscript describing the uptake and efflux studies is in preparation.

Conclusions:

Our initial focus was that drug efflux is an important mechanism which impinges directly on cell survival in response to chemotherapeutic agent treatment. In light of our new and exciting findings, we have now shifted some of our emphasis to understanding drug uptake. This important contribution has the potential to revolutionize how we think about drug resistance. These findings provide impetus for future studies documenting these uptake mechanisms.

Appendices:

None